Microb Ecol (2008) 56:561–571
DOI 10.1007/s00248-008-9376-9

ORIGINAL ARTICLE

Thermally Treated Grass Fibers as Colonizable Substrate for Beneficial Bacterial Inoculum

R. Trifonova · J. Postma · J. J. M. H. Ketelaars ·
J. D. van Elsas

Received: 27 April 2007 / Accepted: 12 February 2008 / Published online: 27 March 2008
© Springer Science + Business Media, LLC 2008

Abstract This study investigates how thermally treated (i.e., torrefied) grass, a new prospective ingredient of potting soils, is colonized by microorganisms. Torrefied grass fibers (TGF) represent a specific colonizable niche, which is potentially useful to establish a beneficial microbial community that improves plant growth. TGF and torrefied grass extracts (TGE) were inoculated with a suspension of microorganisms obtained from soil. Sequential microbial enrichment steps were then performed in both substrates. The microbial communities developing in the substrates were assessed using cultivation-based and cultivation-independent approaches. Thus, bacterial isolates were obtained, and polymerase chain reaction–denaturing gradient gel electrophoresis (DGGE) analyses for bacterial communities were performed. Partial sequencing of the 16S ribosomal RNA gene from isolates and bands from DGGE gels showed diverse communities after enrichment in TGE and TGF. Bacterial isolates affiliated with representatives of the α-proteobacteria (*Methylobacterium radiotolerans*, *Rhizobium radiobacter*), γ-proteobacteria (*Serratia plymuthica*, *Pseudomonas putida*), Cytophaga–Flavobacterium–Bacteroides (CFB) group (*Flavobacterium denitrificans*), β-proteobacteria (*Ralstonia campinensis*), actinobacteria (*Cellulomonas parahominis*, *Leifsonia poae*, *L. xyli* subsp. *xyli*, and *Mycobacterium anthracenicum*), and the firmicutes (*Bacillus megaterium*) were found. In TGE, γ-proteobacteria were dominant (61.5% of the culturable community), and 20% belonged to the CFB group, whereas actinobacteria (67.4%) and α-proteobacteria (21.7%) were prevalent in TGF. A germination assay with lettuce seeds showed that the phytotoxicity of TGF and TGE decreased due to the microbial enrichment.

Introduction

Recently, increased attention has been paid to the recycling of solid organic waste for use as constituents in substrates for ornamental pot plant production [1, 5, 39]. Materials like hardwood, pine barks, composts from different sources, and dried sewage sludge have been considered for this purpose [5, 8, 30, 31], and recently, thermally treated grass has been suggested as a viable alternative.

Torrefaction is applied to protect grass fibers against decay and transform them into a stable product. The process relies on slow heating of grass biomass under anoxic conditions to a maximum temperature of 300°C. The treatment yields a solid product with low moisture content and high energy content per unit mass. Torrefaction can be characterized as “mild pyrolysis,” with removal of gaseous compounds and formation of a solid end product. Approximately 70% of the initial grass fiber weight and 80–90% of the original energy content are retained [26]. Torrefaction leads to enhanced hydrophobicity and phytotoxicity [26] of the grass fibers. It is not clear yet which compounds from the torrefied grass fibers cause the phytotoxic effect. There are data that phenols and phenolic compounds cause phytotoxic problems [3, 28, 38].

R. Trifonova · J. Postma · J. J. M. H. Ketelaars
Plant Research International,
Wageningen, The Netherlands

R. Trifonova · J. D. van Elsas
Microbial Ecology Department,
Centre for Evolutionary and Ecological Studies,
Rijksuniversiteit Groningen,
Haren, The Netherlands

J. Postma (✉)
Droevendaalsesteeg 1,
6708 PB Wageningen, The Netherlands
e-mail: joeke.postma@wur.nl

As the torrefied grass fibers may represent a colonizable niche for microorganisms, it is important to know to which extent they can serve as colonization sites, providing niche space and nutrients such as bound carbon. Alterations in carbon availability will strongly affect microbial community structure and function, which has implications for the interactions with the plants to be grown in the substrate [10]. Multiple limiting nutrients [36], habitat heterogeneity [35], different colonization ability of microorganisms and other factors such as predation and microbial dormancy are responsible for the coexistence of microorganisms of different C-acquiring ability [10]. The types and availability of resources, in particular, available carbon sources, in the TGF and TGE will thus determine the structure of the heterotrophic microbial community that is established and the possible interactions within that community.

In this study, the colonization of TGE and TGF by soil microorganisms was assessed using a sequential enrichment procedure. Cultivation-based and DNA-based methods, i.e., polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE), were applied to assess the microbial communities. A germination assay with lettuce seeds was carried out to analyze phytotoxicity of TGE and TGF.

Methods

Origin of Grass Fibers and Torrefaction Process

Grass material was collected in August 2003 from unfertilized, semi-natural grassland on a peaty clay soil in the vicinity of Wageningen, The Netherlands. The biomass was harvested as a first cut and consisted of a mixture of more than 30 species (mainly grasses, rushes, sedges, and herbs) with a dry matter content of 37.5%. Dry matter content of the plant biomass was measured by drying aliquots at 105°C. The harvested material was dried at 105°C, ground in a hammer mill to pieces of <1 mm, and stored until used for torrefaction experiments.

Samples of the ground material were torrefied in a preheated Carbolite muffle furnace (model CWF 1100) at a temperature of 240°C during 1 h. To ensure the occurrence of anoxic conditions, ground biomass was placed in aluminum boxes (approximately 40 g dry matter in a 200-ml box). The boxes were closed with their lid leaving a narrow split for gas exchange. The boxes, four at a time, were placed upside down in the oven to promote homogeneous heating of the contents. Boxes were weighed before and after heating to determine weight loss of the samples.

Torrefied and untorrefied grass was characterized chemically by the Energy Research Centre of The Netherlands (ECN, Petten, The Netherlands). Contents of C, H, N, and O were analyzed by atomic absorption spectrometry (AAS), and mineral content (Ca, K, Mg, Na, P, and S) was studied by inductively coupled plasma–mass spectrometry (Table 1). Table 2 shows the compounds that were detected by gas chromatography–flame ionization, using a gas chromatograph with a ZB-WAX column (30 m×0.25 mm, *df*= 0.25 μm). The carrier gas was He. The oven was programmed at an initial temperature of 40°C for 5 min with a ramp of 5°C/min to 245°C. Formate was measured by ECN using ion chromatography. Table 2 summarizes the compounds measured in untreated and torrefied grass. Several compounds that were suggested to play a role in phytotoxicity were not found; they were below the detection limit of the applied techniques. These compounds are given in the footnote of the table.

Grass Fiber Extraction

Extracts of the grass fiber matrix were prepared by the following protocol: 9.5 g of torrefied grass fiber was cold-extracted at room temperature with 250 ml distilled water for 2 h using a magnetic stirrer at 700 rpm. After the cold extraction, the suspension was paper-filtered to remove grass fiber particles. The pH was adjusted to 6.8 with KOH. The TGE was then filter-sterilized (Millex GV bacterial filter unit, 0.22-μm, Millipore) and stored at 4°C.

Microbial Inoculum from Soil

To provide a fresh inoculum for inoculation of TGE and TGF, a sandy soil sample was collected from a fireplace exposed some times per year for at least 20 years to high temperature (Wageningen, The Netherlands); this soil was denoted “fireside” soil. It was chosen after a preliminary experiment where two other types of soil, arable soil and forest soil, were used to enrich TGF. However, these enrichments insufficiently removed the phytotoxicity as tested by lettuce seed germination.

A soil suspension from fireside soil was prepared by mixing 10 g of soil with 90 ml 0.1% tetra-sodium pyrophosphate (NaPP–$Na_2P_2O_7.12H_2O$, Merck) and 10 g gravel (2-mm diameter) in 250 ml Erlenmeyer flasks. The flasks were shaken for 30 min at 250 rpm.

Sequential Microbial Enrichment in TGE

Ten milliliters of sterile TGE (pH 6.8) were mixed with 10 ml sterile minimal salts medium (MSM). MSM ingredients were K_2HPO_4 1.4 g/l, KH_2PO_4 1.4 g/l, $MgSO_4.7H_2O$ 0.1 g/l, $(NH_4)_2SO_4$ 1 g/l. Then, 0.2 ml of fireside soil suspension was added to the TGE/MSM mixture. The flasks were incubated for 5 days at 180 rpm, 25°C in a Gallenkamp orbital shaker. After 5 days, an aliquot of 0.2 ml from the

Table 1 Chemical composition of untreated and torrefied (240°C, 1 h) plant biomass

	% of dry matter									
	C	H	N	O	Na	K	Ca	Mg	P	S
Untreated	46.3	5.85	1.17	44.1	0.36	0.55	0.73	0.24	0.10	0.21
Torrefied	51.6	5.45	1.35	35.4	0.42	0.66	0.88	0.30	0.12	0.24

first inoculated flask (denoted N1) was transferred to flask N2, containing 20 ml fresh growth medium TGE/MSM (1:1). Thus, a 100-fold dilution was achieved. The flasks were incubated as described above. This procedure was performed 20 times in two parallel series. At set times, samples were taken for analyses of the microbial communities for PCR–DGGE, colony-forming units (CFU), and phytotoxicity reduction.

Sequential Microbial Enrichment in TGF

The enrichment in TGF was based on an inoculum prepared in a 500-ml Erlenmeyer flask containing 10 g sterile gravel, 10 g fireside soil, and 90 ml MSM medium. The culture was shaken for 30 min at 250 rpm in an orbital incubator (Gallenkamp).

Subsequently, 1.6 ml from the soil suspension was added to 0.5 g of γ-sterilized (25 kGray) TGF in 50-ml vessels, establishing 60% of the substrate's water-holding capacity. The systems were incubated for 7 days at 25°C. After incubation, 5 ml of fresh MSM was added to the 7-day incubated vessels and mixed well. A fresh inoculum of 1.6 ml was transferred from this vessel into new vessels containing 0.5 g sterile TGF, and these novel TGF cultures were again incubated (7 days, at 25°C). The experiment was carried out in two series, and the transfer procedure was repeated 15 times. Samples were taken and analyzed for PCR-DGGE and CFU, and phytotoxicity reduction.

Table 2 Compounds detected by gas chromatography (GC) in untreated and torrefied plant biomass

Compound	Untreated grass mg/kg dry material	Torrefied grass mg/kg dry material
Formate	<20	791
Acetate	185.5	465.6
Hydroxyaceton	12	29.4
2-Furaldehyde	<5	27.9
1-Hydroxy-2-butanon	<5	14.7
Phenol	9.8	11.2
2-Methoxyphenol	3.9	10.8
2,6-Dimethoxyphenol	<10	10.2
Furan-2-methanol	<5	8.6
Pyrrole-2-carboxaldehyde	<5	6.8
Cyclohexanon	<2	3.5

Compounds below the detection limit that is indicated between brackets are propionic acid (<10 mg/kg) 1,2,4-trimethoxybenzene (<5 mg/kg), 2 (5H)-furanon (<10 mg/kg), 2-butenal (<2 mg/kg), 3-methoxypyridine (<10 mg/kg), 3,4,5-trimethoxytoluene (<5 mg/kg), 5-(hydroxymethyl) -2-furaldehyde (<10 mg/kg), 5-methyl-2-furaldehyde (<5 mg/kg), acetaldehyde (<50 mg/kg), acetone (<2 mg/kg), etanol (<5 mg/kg), isoetanol (<10 mg/kg), methanol (<50 mg/kg), methylacetate (<5 mg/kg), methylformate (<2 mg/kg), and propanal (<2 mg/kg)

Phytotoxicity Assay

A lettuce (*Lactica sativa*, cultivar Erika, Enza Zaden, Enkhuizen, The Netherlands) seed germination test was designed to monitor the phytotoxicity of the TGE. Monitoring of enrichment steps 1, 5, 10, 15, and 20 in TGE was done by using 24-well plates (Greiner Bio-one, CELLSTAR®, Germany). Bacterial cells were removed from the TGE enrichments by centrifugation (9.400×*g*, 10 min), and the supernatants were filter-sterilized (Millex GV bacterial filter unit, 0.22 μm, Millipore). Then, 250 μl from the resulting filtrate was used per well. The test was performed in six wells in a randomized design, each with five seeds. After 48 h, the number of seeds that had germinated was counted. As controls, distilled H_2O and TGE/MSM = 1:1 were used.

The seed germination test performed with microbial-enriched TGF was slightly modified from the one in TGE. After 7 days of microbial growth on TGF, 13.2 ml distilled water were added to the fibers, and extraction at room temperature was performed for 2 h using a magnetic stirrer at 700 rpm. The solution was paper-filtered to remove the fibers. Bacterial cells were removed by centrifugation (9.400×*g*, 10 min), followed by filter sterilization as described above. Three treatments were compared: TGF without inoculum (TGF–MO), TGF plus bacterial inoculum transfer 5 (TGF5), and TGF plus bacterial inoculum transfer 15 (TGF15). The samples were kept undiluted and diluted 1:4 and 1:16 in distilled water.

Bacterial Plate Counts

CFU were counted after enrichment steps 1, 3, 5, 10, 15, and 20 in TGE and after enrichment steps 1, 3, 5, 10, and 15 of the sequential enrichment experiment in TGF. Tenfold dilution series were prepared in 0.85% NaCl and plated on R2A medium (Difco, Detroit, MI, USA) supplemented with delvocit (100 mg/l) to inhibit fungal growth. Results are presented as logarithmic numbers of CFU per milliliter TGE and per gram (oven-dry) TGF.

DNA Extraction From TGE and TGF

Total community DNA from enrichment steps 1, 2, 3, 5, 10, 15, and 20 (TGE) and 1, 2, 3, 5, 10, and 15 (TGF) was isolated and purified with the UltraClean™ soil DNA kit (MoBio Laboratories, BIOzymTC, Landgraaf, The Netherlands) according to the protocol of the supplier, except that the cells were disrupted by bead beating four times (30 s each time) in a Braun's cell homogenizer (Braun, Melsungen, Germany) at maximum speed. For disruption, glass beads (50 mg, 0.11 mm diameter) were added to 2 ml of TGE and 0.25 g of TGF, respectively. The bead beating step was included to ensure maximal cell lyses without severe shearing of the DNA. DNA quality and quantity were assessed by electrophoresis in 0.8% agarose gels in 0.5× tris-borate-EDTA buffer [34] by comparison to a standard 1-kb ladder (Invitrogen, Cat.15615-024, Carlsbad, CA, USA). DNA size was 10–40 kb on average.

PCR Amplification

Amplification of 16S ribosomal (r) RNA genes was performed by using PCR in 50-μl reaction volumes containing 0.2 μM of each primer, 3.75 mM $MgCl_2$ (Perkin-Elmer, Nieuwersluis, The Netherlands), 200 μM of each deoxyribonucleotide triphosphate (Boehringer, Almere, The Netherlands) and 0.25 μg of T4 gene 32 protein (Boehringer, Mannheim, Germany) using 5 U Ampli*Taq* Stoffel fragment in Stoffel buffer. The primers used were bacterial 16S rRNA gene forward primer U968 (5′-AACGCGAAGAACCTTAC-3′) and reverse primer R1378 (5′-CGGTGTGTACAAGG CCCGGGAACG-3′) [16]. For DGGE, a GC-clamped version of U968, i.e., U968-GC (5′-CGCCCGGGGCGCGCCCCG GGCGGGGCGGGGGCACGGGGGGAACGCGAAG AACCTTAC-3′) was used. Thermal cycling was as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 65°C for 90 s, 72°C for 2 min, and final extension at 72°C for 10 min. The PCR products (expected sizes about 450 bp) were analyzed by running 5 μl aliquots of the reaction mixtures in 1% agarose gels.

DGGE Analyses

DGGE analyses [15] were performed using 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide 37:1) with a gradient of 45% to 65% denaturants (100% denaturant was defined as 7 M urea plus 40% formamide). The gels were run at 60°C (100 V) for 16 h in a Phor-U2 apparatus (Ingeny International, Goes, The Netherlands) and stained with SYBR gold (Molecular Probes, Leiden, The Netherlands). For analysis of the molecular community profiles, the Molecular Analyst fingerprint software (version 6.0, Biorad, Veenendaal, The Netherlands) was used.

Recovery, Purification, and Sequencing of DNA Template From DGGE Bands

Organisms underlying the DGGE profiles were analyzed by reamplifying and sequencing bands of interest. DNA was eluted from excised bands as described by Salles [33]. Crushed excised bands were incubated in 20 μl of sterile H_2O at 65°C for 30 min, followed by centrifugation at 5,000×*g* for 1 min. The eluted DNA was used as the template for PCR amplification. Selected bands were reamplified using the clamped PCR system and run again on DGGE gel. Only bands with migratory positions identical to those originally found were used for subsequent DNA sequence analysis.

Purified PCR products were subjected to a final cleaning step using the high-pure PCR purification kit (Roche GmbH, Mannheim, Germany) and then sequenced using an ABI Prism automatic sequencer (Greenomics, Wageningen, The Netherlands). The partial 16S rRNA gene sequences were compared with those available in the National Center for Biotechnology Information (NCBI) database using BLAST-N. The sequences generated were deposited in the GenBank under the accession numbers EU287845 to EU287853.

Identification and Sequencing of Bacterial Strains

In total, 111 bacterial strains were isolated and maintained as pure cultures on R2A medium at 20°C. Long-term storage in glycerol stocks at −70°C crude DNA was obtained from all 111 strains as described by Salles [32]. Briefly, one freshly grown colony per strain was resuspended in 20 μl of sterile Milli-Q water in a microfuge tube. Cells were lysed by keeping the suspension at 95°C for 10 min, followed by cooling on ice for 10 min. Before performing lyses, 5 mg of Chelex-100 resin (Sigma-Aldrich BV, Zwijndrecht, The Netherlands) was added to the microtubes. By this procedure, inhibitory compounds released from lysing cells were sequestered by the resin, and subsequent centrifugation (13.400×*g*, 5 min) removed them from the suspension. The microfuge tubes containing the crude lysates were stored at −20°C.

The crude lysates of the strains were used as a template for PCR reaction for sequencing. This was done as it was described in section PCR amplification. The PCR products were purified with High-pure PCR purification kit (Roche

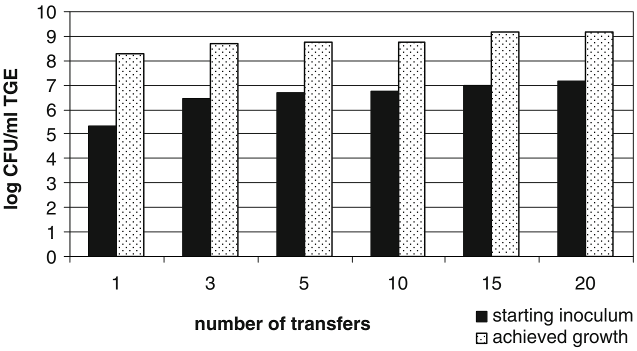

Figure 1 Bacterial numbers (log CFU/ml) of starting inoculum and achieved growth after 5 days of colonization in sequential enrichment steps in TGE. Least significant difference is 1.43 (p=0.05)

GmbH Mannheim, Germany) and then sequenced using an ABI Prism automatic sequencer (Greenomics, Wageningen, The Netherlands).

The partial 16S rRNA gene sequences were investigated by DNAstar v6/Seqman program and compared with those available in the NCBI database using BLAST-N. The sequences generated were deposited in the GenBank under the accession numbers EU293366 to EU293390. As many of the strains had a repeatable hit in the NCBI database (BLAST-N), only one representative sequence of each group of strains was send to the GenBank.

Carbon Substrate Utilization Test

The 111 strains isolated from the TGE and TGF enrichment experiment were grouped in 17 groups by BOX-PCR (data not shown). Representative strains from each group were tested in duplicate for growth on the main readily available carbon sources in TGF, acetic acid, and potassium formate. Bacterial colonies grown on R2A were inoculated into Erlenmeyer flasks (50 ml) containing MSM, as described previously, supplemented with either acetic (1%) or formic acid (1%) and 0.001% yeast extract, or a combination of both carbon sources, i.e., 0.5% acetic acid and 0.5% K-formate, and 0.001% yeast extract. After adjustment of the pH to 6.9, the solutions were filter-sterilized (Millex GV bacterial filter unit, 0.22 µm, Millipore) before bacterial inocula were added. The Erlenmeyer flasks were shaken at 25°C (180 rpm). Bacterial growth was recorded by increase of OD_{600}, using a Beckman DU® 530 Life Science UV/Vis spectrophotometer at regular time intervals, i.e., on days 2, 4, 6, 8, and 10. $OD_{600}>0.1$ was regarded as positive.

Statistical Analyses

Analyses of variance (ANOVA) were performed for CFU enumerations and germination data with the statistical program Genstat 8 (Rothamsted Experimental Station, Harpenden, UK). Least significant differences were calculated at a significant level of P=0.05. The CFU data were expressed as mean values over triplicate experimental units and transformed to logarithmic values. The germination percentages were analyzed by two-way ANOVA: The significance of the enrichment step and replicate series were analyzed in TGE, and the significance of the enrichment step and the dilution were analyzed in TGF.

Cluster analyses of DGGE patterns were performed by GelCompar II (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Dendrograms were generated based on the unweighted pair group method with arithmetic mean and Pearson correlation that represented the similarities between the PCR–DGGE profiles.

Results

Bacterial Plate Counts

The bacterial numbers increased from log 5.2 (starting inoculum) up to log 8.2 (after the first enrichment step) and showed a successive increase to log 9.2 per milliliter in the last enrichment steps (15 and 20; Fig. 1). There was no significant difference between replicate series.

The number of bacteria initially introduced onto the TGF was log 8.4 CFU/g (Fig. 2). After the first transfer, the CFU number reached log 9.1 per gram. CFU numbers increased during the ensuing enrichment stages up to log 10.2 per gram in transfer 15. There was no significant difference between replicate series.

Phytotoxicity Assay

Lettuce seed germination in TGE was significantly enhanced by the microbial enrichment. TGE without microbial colonization (untreated TGE/MSM = 1:1) only yielded 3% germination, whereas the percentage of germinated

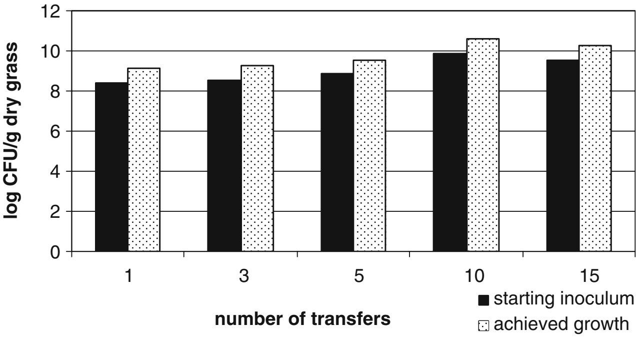

Figure 2 Bacterial numbers (log CFU/g) of starting inoculum and achieved growth after 7 days of colonization in sequential enrichment steps in TGF. Least significant difference is 0.32 (p=0.05)

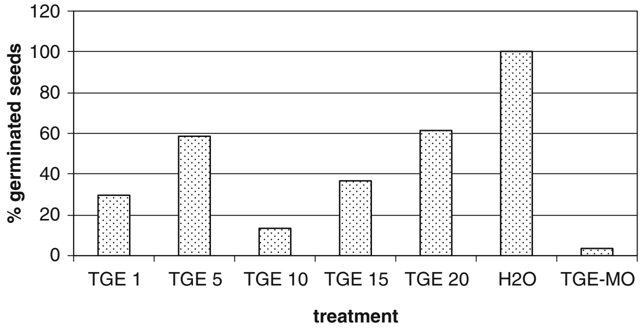

Figure 3 Lettuce seed germination after sequential microbial enrichment steps in TGE, compared to germination in a water control and TGE without microorganisms (TGE-MO). Least significant difference is 18.2 (*p*=0.05)

seeds varied between 17% and 62% in the enriched TGE (Fig. 3). Germination was improved significantly in TGE1, TGE5, TGE15, and TGE20 compared to the uninoculated control. The two replicate series were not significantly different (*P*>0.05). In the water control, all seeds germinated.

No seeds germinated in the treatments TGF without MO and TGF5. In contrast to these results, TGF15 showed 26.7% germination (Fig. 4). Dilutions of 1:4 and 1:16 were included for all three treatments to obtain better contrasts in the phytotoxic assay of TGF. Using 1:4 and 1:16 dilution for TGF5, the germination was increased up to 76.7% and 100%, respectively, whereas seed germination in TGF without MO was only 63% and 75%, respectively. TGF15 gives always significantly higher percentage of germination compared to uncolonized TGF.

DGGE Fingerprints of TGE and TGF

The bacterial community colonizing TGE showed different and shifting levels of complexity during the subsequent enrichment steps (Fig. 5a). In TGE1, the banding patterns were complex and multiple, showing at least 17 bands. At increasing enrichment steps, fewer bands occurred, and these became more pronounced. The banding patterns in both replicates of every enrichment step, except for 1a and 1b, had virtually the same number of bands and position, which confirms the consistency between replicates. In TGE2, TGE3, and TGE5, approximately 14 bands were present. Thereafter, in TGE10, TGE15, and TGE20, the number of bands had decreased and a balanced community with a total of about ten bands had developed.

Few bands (Fig. 5a; bands 1, 2, and 4) dominated in the patterns between the TGE2 and TGE20 enrichment step. This may indicate the selection of a specific bacterial type as a result of substrate or ambient adaptation.

A dendrogram was constructed based on band composition (Fig. 5b). All replicates (except in 1a, 1b and 3a, 3b) were clustered together, showing relatively low interreplicate variability. Two large clusters were found, with similarity levels between them of 60%. One cluster included all samples from 10 to 20, whereas the other one encompassed all samples from 2 to 5. Samples 1a and 1b were quite different from all the other samples. Within the two main clusters, smaller sub-clusters in accordance with transfer number (both higher than 70% similarity) were observed. Figure 6a shows the DGGE bacterial fingerprints obtained from the TGF samples. The bacterial communities apparently reached stability after five transfers, as it can be seen from the convergent profiles obtained in the enrichment steps 5, 10, and 15. Enrichment step 5 thus seems crucial because the main bacterial selection occurred around this phase. There were few very strong bands (Fig. 6a, bands 5 and 6), which appeared as from the third enrichment step and were present until the end of the experiment. Consistency of number and location of bands between the two replicates was observed during every enrichment step. After enrichment step 1, the profiles consisted of many bands, occurring close to each other. This created difficulties to count their exact number. After enrichment step 2, about 20 bands were counted for both replicates, while after enrichment step 3, the number of bands was reduced to up to 14 with a slight difference in band numbers between the two replicates. After enrichment step 5, a clear banding pattern consisting of 12 bands emerged. This pattern remained consistent throughout the next enrichment steps, i.e., 10 and 15. One band (Fig. 6a, band 7), which was present in enrichment steps 1, 2, 3, and 5, disappeared in enrichment steps 10 and 15.

According to the dendrogram (Fig. 6b), two clear clusters were formed, enrichments 1, 2, and 3 with similarity of the profiles of about 94% and enrichments 5, 10, and 15 with 96% similarity. The main clusters were each divided in sub-clusters that tended to group replicates together.

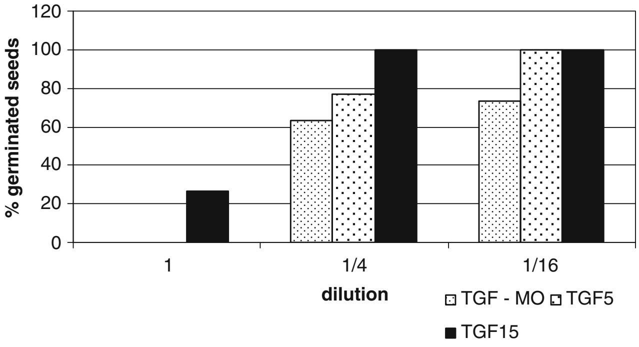

Figure 4 Lettuce seed germination after sequential microbial enrichment steps in TGF, compared to germination in TGF without microorganisms (TGE-MO). The treatments were tested undiluted (*1*) and diluted 4 and 16 times. Least significant difference is 18.9 (*p*=0.05)

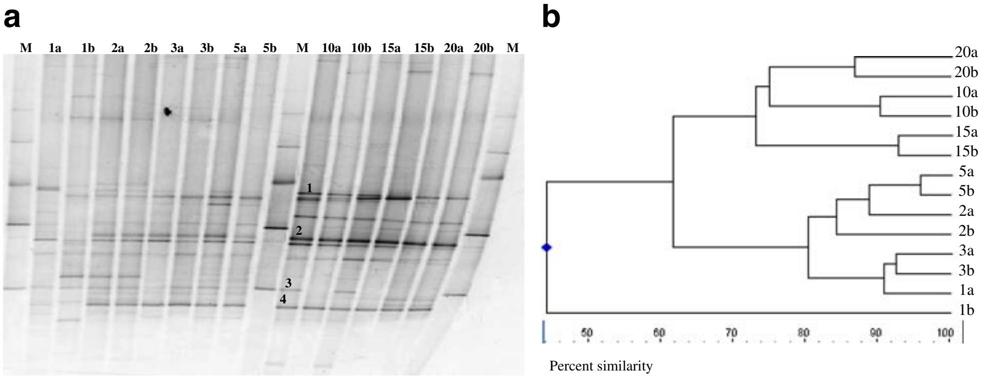

Figure 5 **a** PCR-DGGE banding patterns of torrefied grass extract at different sequential enrichment steps in duplicate series a and b. *M* is a bacterial marker. *Numbers* in the gel represent sequenced bands. **b** A dendrogram representing the similarity of PCR–DGGE profiles generated with bacterial DNA from different enrichment steps in TGE

Identification of Bands in PCR–DGGE Patterns From TGE and TGF

Selected bands present in TGE (Fig. 5a, Table 3, bands 1, 2, 3, and 4) and TGF (Fig. 6a, Table 3, bands 5, 6, 7, and 8) fingerprints were excised, reamplified, and sequenced. A database analysis revealed that the closest matches to the bands in TGE encompassed a bacterium belonging to the Cytophaga–Flavobacterium–Bacteroides (CFB), i.e., *Flavobacterium denitrificans* (97.7%, band 1), and three bacteria belonging to γ-proteobacteria, i.e., *Stenotrophomonas maltophilia* (95.5% band 2) *Pseudomonas gingeri* (98.7% band 3), and *P. putida* (98.7% band 4).

In the TGF system, we found evidence for the occurrence of strains affiliated with *Afipia broomeae* (98%, band 5, α-proteobacteria) and *Arthrobacter chlorophenolicus* (97.6%, band 6, actinobacteria), *Agromyces aurantiacus* (95%, band 7), and *Streptomyces paresii* (99.1%, band 8).

Identification of Bacterial Isolates

In total, 111 bacterial strains were isolated from the sequential enrichments, respectively 37, 28, 12, and 34 from TGE5, TGE20, TGF10, and TGF15. The sequencing results show that the most abundant strain in TGE5 was affiliated to *P. plecoglossicida*, while *F. denitrificans* dominated in TGE20 (Table 4).

The bacterial communities in TGF10 and TGF15 consisted mainly of Gram-positive bacteria (Table 4). The most frequently found strain in TGF15 was identified as *Leifsonia poae*. Other less dominating strains in TGF15 were *Mycobacterium anthracenicum* and *L. xyli* subsp. *xyli*. In TGF10, *Bacillus megaterium*, *Methylobacterium radiotolerans*, and *L. xyli* subsp. *xyli* shared almost the same number of strains (three, three, and four, respectively). *Cellulomonas parahominis* and *Tsukamurella poriferae* were less frequently found.

The partial 16S rRNA analysis of the isolates from the different enrichment steps on TGE and TGF showed bacterial communities of limited diversity. Bacteria adapted to TGE were mainly Gram-negative in contrast with the ones isolated from TGF, which were mainly Gram-positive. Taking both TGE5 and TGE20 into account, 61.5% belonged to γ-proteobacteria, 20% to the Cytophaga–Flavobacterium–Bacteroides, 6.2% to β-proteobacteria, 9.2% to the actinobacteria, and 3.1% to the α-proteobacteria.

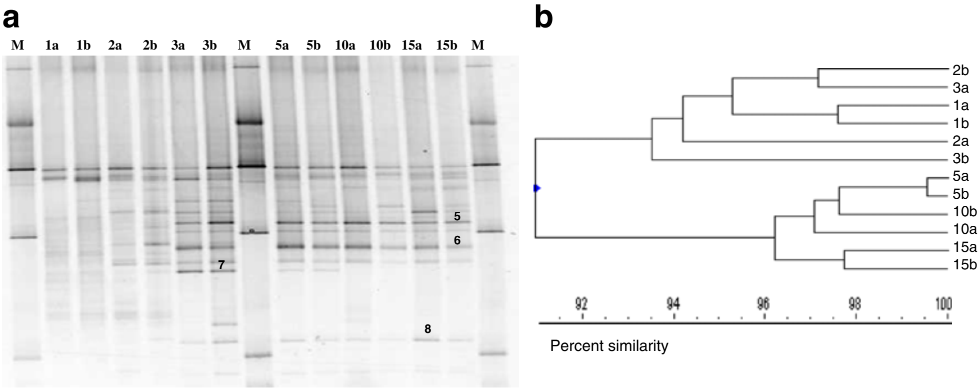

Figure 6 **a** PCR–DGGE banding pattern of torrefied grass fiber (TGF) after different sequential enrichment steps in duplicate series a and b. *M* is a bacterial marker. **b** A dendrogram representing the similarity of PCR–DGGE profiles generated with bacterial DNA from different enrichment steps in TGF

Table 3 Closest matches for 16S rRNA gene sequences of bands excised from PCR–DGGE banding patterns of TGF and TGE

Band origin	Closest relative in GenBank	Percentage similarity	GenBank accession no.	Reference	Closest type strain	Percentage similarity	Type strain GenBank accession no.	Reference
1TGE	*Flavobacterium denitrificans.*	97.7	AJ318907	[12]	*Flavobacterium denitrificans*	94.6	AJ704214	[9]
2TGE	*Stenotrophomonas maltophilia;*	95.5	EU263112	–	*Stenotrophomonas maltophilia*	94.7	AB008509	[13]
	Uncultured alpha proteobacterium	96	AM114434	[5]				
3TGE	*Pseudomonas gingeri*	98.7	AF320991	[7]	*Pseudomonas pictorum*	94.7	AB021392	[2]
4TGE	*Pseudomonas putida/ Pseudomonas plecoglossicida*	98.7/98.9	DQ229315/ AJ245436	–/[14]	*Pseudomonas plecoglossicida*	98.7	AB009457	[23]
5TGF	*Afipia broomeae*	98	AY568506	–	*Afipia massiliensis*	98	AY029562	[17]
6TGF	*Arthrobacter chlorophenolicus*	97.6	AF102267	[40]	*Arthrobacter chlorophenolicus*	97.6	AF102267	[40]
7TGF	*Agromyces aurantiacus*	95	AF389342	[4]	*Agromyces aurantiacus*	95	AF389342	[19]
8TGF	*Streptomyces paresii*	99.1	AJ969177	–	*Streptomyces atroolivaceus*	98.5	AJ781320	[18]

In contrast, isolates from TGF10 and TGF15 belonged, for 67.4%, to actinobacteria, 6.5% to firmicutes, and 21.7% α-proteobacteria, and only 4.3% were γ-proteobacteria.

Comparing bands identified in the TGE and TGF systems (Table 3) with the identified strains in TGE and TGF (Table 4), there was a clear match between most of the identified bands with some of the isolated bacteria. For example, *F. denitrificans*, *S. maltophilia*, and *P. putida* were found as bands in TGE derived from DGGE profiles and also as isolates from TGE. A similar link was observed for *Afipia broomeae* and *Agromyces aurantiacus*. Those species were found as bands and also as isolates from TGF. However,

Table 4 Closest relatives of the isolates from TGE and TGF

The closest related bacterial sequence	Accession no	% identity	Numbers of strains isolated from TGE5	Numbers of strains isolated from TGE20
Arthrobacter nicotinovorans	AY833102	99.5	6	–
Flavobacterium denitrificans	AJ318907	99.2–91.7	3	10
Pseudomonas corrugata	EF153018	99.7	1	–
Pseudomonas putida	DQ229317	99.2	3	6
Pseudomonas plecoglossicida	DQ095892	96	17	7
Ralstonia campinensis	AF312020	97.6	1	3
Rhizobium radiobacter	AY626383	98.6	–	2
Serratia plymuthica	AY 551332	100	2	–
Stenotrophomonas maltophilia	AY841799	99.5–97.5	4	–
			Numbers of strains isolated from TGF10	Numbers of strains isolated from TGF15
Agrococcus casei	DQ168427	98.7	–	1
Agromyces aurantiacus	AF389342	97.2	–	1
Afipia broomeae	U87760	97.1	–	2
Bacillus megaterium	DQ207561	99.8	3	–
Cellulomonas parahominis	AY655729	99.7	1	3
Leifsonia poae	AM410682	97.7	–	16
Leifsonia xyli subsp. xyli	DQ232616	98.7	4	4
Mycobacterium anthracenicum	MSY15709	98.4	–	7
Methylobacterium radiotolerans	AY616142	99.5–94.4	3	–
Tsukamurella poriferae	AY714240	100	1	–

Streptomyces was detected in the DGGE banding pattern of TGF but was not present among the isolated bacteria.

Carbon Substrate Utilization

Out of the 17 strains tested, only four strains, denoted 4, 23, 69, and 70, grew on MSM medium supplemented with acetic acid as sole carbon source (plus 0.001% yeast extract), whereas only two strains (56 and 70) grew on MSM medium supplemented with 1% K-formate (plus 0.001% yeast extract). In contrast, 16 out of 17 strains grew well on MSM medium supplemented with 0.5% acetic acid and 0.5% K-formate, in the presence of 0.001% yeast extract (Table 5). The bacteria in the yeast extract itself did not spur growth.

Discussion

In this study, TGE and TGF were inoculated with a microbial inoculum obtained from soil to assess which soil microorganisms can stably colonize these systems. For that purpose, sequential microbial enrichments were performed. Using the enrichments, we aimed to select those microorganisms that might be adapted to the matrix and/or its carbonaceous compounds. After 20 sequential steps of enrichment in TGE and 15 in TGF, which was estimated to be equivalent to 140 and 43 generations, respectively, presumably microorganisms adapted to the prevailing conditions in the two systems were selected, and knowledge was obtained regarding the community compositions. Table 2 shows 11 compounds that were present after the torrefaction. Some of these compounds may serve as easily available carbon source for many bacteria. Two of these occurred in amounts that could potentially spur considerable microbial growth, i.e., acetate (465.6 mg/kg) and formate (791 mg/kg). As we assumed, the selected strains could grow on minimal media supplied with acetate and formate as sole carbon sources. Sixteen out of 17 isolates grew on the combination of both carbon sources. However, only few strains utilized formate and acetate separately. Some of the other compounds, namely 2-furaldehyde (27.9 mg/kg), phenol (11.2 mg/kg), 2-methoxyphenol (10.8 mg/kg), 2,6-dimethoxyphenol (10.2 mg/kg), and furan-2-methanol (8.6 mg/kg) might be involved in phytotoxicity but could also serve as a nutrient source.

The CFU on TGF were high (10^9–10^{10} per gram) in comparison with other organic substrates such as potting soil or compost, which contain 10^8–5×10^9 bacterial CFU [27]. The bacterial numbers in TGF always increased from a diluted inoculum to more or less the same high level. This is an indication that torrefied grass fibers have a large carrying capacity. In other words, they offer specific colonizable niches for microorganisms and provide substrates and space for microbial development. In this sense, fiber's carrying capacity is the amount of microorganisms that could live on the torrefied grass fibers. As a consequence, the fibers are expected to be a good substrate for microorganisms. The clear reduction of number of

Table 5 Growth of different strains from TGE and TGF in acetic acid, K-formate and the combination of both as C-source in a minimal salt solution with 0.001% yeast extract

Strain number	Origin	Closest related sequence	1% acetic acid[a]	1% K-formate[a]	0.5% ac.a + 0.5%K-formate[a]
4	TGE5	*Pseudomonas plecoglosicida*	+	−	+
15	TGE5	*Pseudomonas putida*	−	−	+
23	TGE5	*Serratia plymutica*	+	−	+
26	TGE5	*Flavobacterium denitrificans*	−	−	+
31	TGE5	*Pseudomonas corrugata*	−	−	+
34	TGE5	*Stenotrophomonas maltophilia*	−	−	+
42	TGE20	*Ralstonia campinensis*	−	−	+
43	TGE20	*Rhizobium radiobacter*	−	−	+
56	TGF10	*Methylobacterium radiotolerans*	−	+	+
66	TGF10	*Leifsonia xyli subsp. xyli*	−	−	+
68	TGF10	*Bacillus megaterium*	−	−	+
69	TGF10	*Cellulomonas parahominis*	+	−	+
70	TGF15	*Mycobacterium anthracenicum*	+	+	+
72	TGF15	*Agrococcus casei*	−	−	+
82	TGF15	*Leifsonia poae*	−	−	−
94	TGF15	*Afipia broomeae*	−	−	+
95	TGF15	*Agromyces aurantiacus*	−	−	+

Plus sign $OD_{600}\geq0.1$, *minus sign* $OD_{600}\leq0.1$

[a] Based on measurements of two replicates

bands on DGGE during the enrichment steps, in both systems TGE and TGF, indicated that the bacterial community was increasingly adapted to the TGE and TGF environment over transfer steps. Moreover, all DGGE fingerprints obtained from TGE and TGF over time showed considerable differences between the two systems. This was understandable in ecological terms, as the physical nature of the substrate was different, i.e., a liquid, well-aerated system in the case of TGE versus a solid-dominated and possibly locally oxygen-limited system in the case of TGF. Hence, in our selection procedure, we mimicked two sets of widely divergent ecological conditions, which in turn were selective for two divergent microbial community structures. Sequencing analyses of bands from TGE and TGF revealed phylogenetic differences. Cytophaga–Flavobacterium–Bacteroides (CFB) and γ-proteobacteria were typical for TGE, and α-proteobacteria and actinobacteria were found in TGF. The differences in microbial groups might be also related to the differences in the physical nature of both substrates.

Intriguingly, consistent and stable patterns of number and position of bands were achieved after few enrichment steps in both systems. The communities in TGE and TGF ended up with about ten bands, an indication that the community contains several bacteria living together on the same substrate. Few bands (Fig. 5a) found in the fingerprint of the first sequential enrichment, disappeared in later enrichment steps, possibly because of poor adaptation of the underlying organism to the applied conditions.

Comparing the bands from TGE and TGF DGGE fingerprints in the final enrichment steps with the sequences of the isolates obtained from both systems, almost all bands from the TGE and some from the TGF had a corresponding strain. However, *Streptomyces* was not present among the isolated bacteria.

Interestingly, several sequences of bands as well as isolates corresponded with sequences from isolates originating from rather extreme, sometimes-toxic environments. For example, the sequence determined for band 2 identified as remotely related to *S. maltophilia* was discovered in uranium mining waste, in which heavy metals and radionuclides abound [6]. In total, nine isolates from TGE5 and TGE20 were affiliated with *P. putida* (99.2%) that, according to the NCBI database, was shown to be tolerant to (and possibly consuming) toluene. Isolates from TGF10 were identified as akin to *B. megaterium* (99.8%), and this strain was obtained from “naphtha” water transporting pipelines [29]. Interestingly, band 6 was identified as *Arthrobacter chlorophenolicus*, a species capable of degrading high concentrations of 4-chlorophenol [25]. Band 5 affiliated with *A. broomeae*, which was similar to a sequence found in radioactive waste [22]. *Flavobacterium* is known as a bacterium-utilizing furfural [20]. *Methylobacterium* possesses rather diverse characteristics from producing 1-aminocyclopropane-1-carboxylate deaminase [11, 21] to radioresistance [24] and Zn resistance [16]. The fact that members of *Methylobacterium* and *Flavobacterium* were found might give an indication that these bacteria were selected for their use of toxic and derivatives present in the TGE and TGF.

As a corollary, we obtained evidence for the selection of microbial communities able to degrade phytotoxic compounds via lettuce seed germination test. This test assesses whether the level of phytotoxic compounds decreases as a result of microbial growth by measuring increase of seed germination. The communities enriched in TGE as well as in TGF were able to remove the phytotoxicity. In further research (unpublished data) [37], it was proved that the bacterial strains *S. maltophilia* (isolate 34), *P. putida* (isolate 15), *Serratia plymuthica* (isolate 23), *P. corrugata* (isolate 31), *M. radiotolerans* (isolate 56), and *Agromyces aurantiacus* (isolate 95) could decrease the concentration of some of the toxic compounds present in TGF, such as 2-furaldehyde and pyrrole-2-carboxaldehyde but not the phenol, 2-methoxyphenol, and 2,6-dimethoxyphenol.

The above information is, to a certain degree, consistent with the expectation that degradable compounds that cause phytotoxicity might be transformed by the bacterial community selected. To deal with the phytotoxic compounds from TGE and TGF, bacteria should possess specific mechanisms to either break down or tolerate potentially toxic compounds. Moreover, we focused our attention on the bacteria, but other microorganisms such as fungi could also be important for diminishment of toxicity.

This investigation on the colonization of TGE and TGF systems represents the first study that addresses the settlement of bacteria on torrefied grass fibers. After many enrichment steps, the final communities in both systems consisted of approximately ten dominant bacterial species growing together on the substrates. Moreover, the communities of TGE and TGF were rather different. A decrease in phytotoxicity due to the microbial enrichment was observed in TGE as well as in TGF. However, many questions still remain. For instance, which bacteria present in the enriched community are involved in reducing phytotoxicity, and can they be applied to TGF to obtain a substrate suitable for plant growth?

Acknowledgment This research was supported by the Dutch Ministry of Agriculture, Nature and Food Quality.

References

1. Abad M, Noguera P, Puchades R, Maquieira A, Noguera V (2002) Physico-chemical and chemical properties of some coconut coir dusts for use as a peat substitute for containerised ornamental plants. Bioresour Technol 82:241–245

2. Anzai Y, Kim H, Park JY, Wakabayashi H, Oyaizu H (2000) Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. Int J Syst Evol Microbiol 50:1563–1589
3. Aranda E, Sampedro I, Ocampo JA, Garcia-Romera I (2006) Phenolic removal of olive-mill dry residues by laccase activity of white-rot fungi and its impact on tomato plant growth. Int Biodeterior Biodegrad 58:176–179
4. Cui XL, Mao PH, Zeng M, Li WJ, Zhang LP, Xu LH, Jiang CL (2001) *Streptimonospora salina gen. nov.*, sp. *nov.*, a new member of the family Nocardiopsaceae. Int J Syst Evol Microbiol 51:357–363
5. Fornes F, Belda RM, Abad M, Noguera P, Puchades R, Maquieira A, Noguera V (2003) The microstructure of coconut coir dusts for use as alternatives to peat in soilless growing media. Aust J Exp Agric 43:1171–1179
6. Geissler A, Selenska-Pobell S (2005) Addition of U(VI) to a uranium mining waste sample and resulting changes in the indigenous bacterial community. Geobiology 3:275–285
7. Godfrey SAC, Marshall JW, Klena JD (2001) Genetic characterization of *Pseudomonas* '*NZI7*'—a novel pathogen that results in a brown blotch disease of *Agaricus bisporus*. J Appl Microbiol 91:412–420
8. Handreck KA, Black ND (1993) Growing media for ornamental plants and turf, 2nd edn. New South Wales University Press, Kensington
9. Horn MA, Drake HL, Schramm A (2006) Nitrous oxide reductase genes (nosZ) of denitrifying microbial populations in soil and the earthworm gut are phylogenetically similar. Appl Environ Microbiol 72:1019–1026
10. Hu S, van Bruggen A, Grunwald N (1999) Dynamics of bacterial populations in relation to carbon availability in a residue-amended soil. Appl S Ecol 13:21–30
11. Idris R, Trifonova R, Puschenreiter M, Wenzel WW, Sessitsch A (2004) Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. Appl Environ Microbiol 70:2667–2677
12. Ihssen J, Horn MA, Matthies C, Gossner A, Schramm A, Drake HL (2003) N2O-producing microorganisms in the gut of the earthworm *Aporrectodea caliginosa* are Indicative of ingested soil bacteria. Appl Environ Microbiol 69:1655–1661
13. Iizuka T, Yamanaka S, Nishiyama T, Hiraishi A (1998) Isolation and phylogenetic analysis of aerobic copiotrophic ultramicrobacteria from urban soil. J Gen Appl Microbiol 44:75–84
14. Kok M, Oldenhuis R, van der Linden M, Raatjes P, Kingma J, van Lelyveld P, Witholt B (1989) The *Pseudomonas oleovorans* alkane hydroxylase gene. Sequence and expression. J Biol Chem 264:5435–5441
15. Kowalchuk GA, de Bruijn FJ, Head IM, Akkermans AD, van Elsas JD (2004) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. Molecular microbial ecology manual II, vol. 2. Kluwer, Dordrecht, The Netherlands, pp 743–789
16. Kunito T, Shibata S, Matsumoto S, Oyaizu H (1997) Zinc resistance of *Methylobacterium* species. Biosci Biotech Biochem 61:729–731
17. La Scola B, Mallet MN, Grimont PAD, Raoult D (2002) Description of *Afipia birgiae* sp. *nov.* and *Afipia massiliensis* sp. *nov.* and recognition of *Afipia felis* genospecies A. Int J Syst Evol Microbiol 52:1773–1782
18. Lanoot B, Vancanneyt M, Hoste B, Vandemeulebroecke K, Cnockaert MC, Dawyndt P, Liu Z, Huang Y, Swings J (2005) Grouping of streptomycetes using 16S-ITS RFLP fingerprinting. Res Microbiol 156:755–762
19. Li W-J, Zhang L-P, Xu P, Cui X-L, Xu L-H, Zhang Z, Schumann P, Stackebrandt E, Jiang C-L (2003) *Agromyces aurantiacus* sp. *nov.*, isolated from a Chinese primeval forest. Int J Syst Evol Microbiol 53:303–307
20. López MJ, Nichols NN, Dien BS, Moreno J, Bothast RJ (2004) Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. Appl Microb Biotech 64:125–131
21. Madhaiyan M, Kim BY, Poonguzhali S, Kwon SW, Song MH, Ryu JH, Go SJ, Koo BS, Sa TM (2007) *Methylobacterium oryzae* sp *nov.*, an aerobic, pink-pigmented, facultatively methylotrophic, 1-aminocyclopropane-L-carboxylate deaminase-producing bacterium isolated from rice. Int J Syst and Evol Microb 57:326–331
22. Nedelkova M (2005) Microbial diversity in groundwater at the deep-well monitoring site S15 of the radioactive waste depository Tomsk-7, Siberria, Russia, Freiberg, Germany
23. Nishimori E, Kita-Tsukamoto K, Wakabayashi H (2000) *Pseudomonas plecoglossicida* sp. *nov.*, the causative agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis*. Int J Syst Evol Microbiol 50:83–89
24. Nogueira F, Luisa Botelho M, Tenreiro R (1998) Radio-resistance studies in *Methylobacterium* spp. Radiat Phys Chem 52:15–19
25. Nordin K, Unell M, Jansson JK (2005) Novel 4-chlorophenol degradation gene cluster and degradation route via hydroxyquinol in *Arthrobacter chlorophenolicus* A6. Appl Environ Microbiol 71:6538–6544
26. Pach MZR, Björnbom E (2003) Presented at the 6th Asia-Pacific International Symposium on Combustion and Energy Utilisation, 20–22 May 2003, Kuala, Lumpur, Malaysia
27. Postma J, Montanari M, van den Boogert, Paul H (2003) Microbial enrichment to enhance the disease suppressive activity of compost. Eur J Soil Biol 39:157–163
28. Quaratino D, D'Annibale A, Federici F, Cereti CF, Rossini F, Fenice M (2007) Enzyme and fungal treatments and a combination thereof reduce olive mill wastewater phytotoxicity on *Zea mays* L. seeds. Chemosphere 66:1627–1633
29. Rajasekar APS, Maruthamuthu S, Palaniswamy N, Rajendran A (2006) Presented at the Recent Advances in Marine Autifouling Technology (RAMAT), 5–8 November 2006, Sagar Sangamam, India
30. Raviv M (1998) Horticultural uses of composted material. Acta Hortic 469:225–234
31. Ribeiro HMEV, Dos Santos JQ (2000) Fertilisation of potted geranium with a municipal solid waste compost. Bioresour Technol 73:247–249
32. Salles JF, Elsas JDv, van Veen JA (2006) Effect of agricultural management regime on *Burkholderia* community structure in soil. Microb Ecol 52:267–279
33. Salles JF, van Veen JA, van Elsas JD (2004) Multivariate analyses of *Burkholderia* species in soil: effect of crop and land use history. Appl Environ Microbiol 70:4012–4020
34. Sambrook J, Fritsch EF, Maniatis T (1998) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, USA, NY
35. Smiles D (1988) Aspects of the physical environment of soil organisms. Biol Fertil Soils 6:204–215
36. Tilman D (1982) Resource competition and community structure. Princeton University Press Princeton, NJ, USA
37. Trifonova R, Postma J, Francel V, Bouwmeester H, Ketelaars JJMH, Van Elsas JD (2008) Removal of phytotoxic compounds from torrefied grass fibres by microorganisms. FEMS Microb Ecol (in press)
38. Ucisik AS, Trapp S (2006) Uptake, removal, accumulation, and phytotoxicity of phenol in willow trees (*Salix viminalis*). Environ Toxicol Chem 25:2455–2460
39. Verdonck O (1984) Reviewing and evaluation of new material used as substrates. Acta Hortic 150:467–473
40. Westerberg K, Elvang AM, Stackebrandt E, Jansson JK (2000) *Arthrobacter chlorophenolicus* sp. *nov.*, a new species capable of degrading high concentrations of 4-chlorophenol. Int J Syst Evol Microbiol 50:2083–2092